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TOXICITY OF THE CYCLIC NITRAMINE ENERGETIC MATERIAL CL-20 TO AQUATIC RECEPTORS

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14. ABSTRACT
We investigated the effects of the cyclic nitramine energetic material CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane) on *Ceriodaphnia dubia* (water flea), *Pimephales promelas* (fathead minnow), and *Selenastrum capricornutum* (green single celled algae). We designed experiments to include two types of exposure: (1) direct amendments of CL-20 to test media, and (2) aquatic elutriates from Sassafras Sandy Loam (SSL) soil amended with various concentrations of CL-20. Selected exposure routes that we simulated for testing included the potential toxicity of CL-20 to aquatic receptors resulting from direct CL-20 release into the aquatic environment, and from contaminated surface soil runoff. CL-20 concentrations in aquatic media were determined using a modified USEPA Method 8330, then correlated with reproduction or growth endpoints using nonlinear regression models to determine the respective IC_{20} and IC_{50} values for CL-20. Experiments using direct amendments of CL-20 to test media resulted in IC_{20} values of 2.0 mg L^{-1} for P. promelas (growth), 1.2 mg L^{-1} for C. dubia (reproduction), and 31 mg L^{-1} for S. capricornutum (growth). In experiments using elutriates from SSL soil amended with CL-20 resulted in IC_{20} values of 1.4 mg L^{-1} for P. promelas (growth), 1.1 mg L^{-1} for C. dubia (reproduction) and >8 mg L^{-1} for S. capricornutum (growth).

15. SUBJECT TERMS

CL-20	*Ceriodaphnia dubia*	Aquatic Toxicity	Soil amendment
Selenastrum capricornutum	Fathead minnow	Soil extract	

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PREFACE

The work described in this report was authorized under the Strategic Environmental Research and Development Program (SERDP CP-1254). This work was started in October 2002 and completed in March 2005.

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Acknowledgment

This work was conducted in cooperation with and funded by the Strategic Environmental Research and Development Program (SERDP CP-1254).

QUALITY ASSURANCE

This study, as reported by "Toxicity of the Cyclic Nitramine Energetic Material CL-20 to Aquatic Receptors", was examined for compliance with Good Laboratory Practices as published by the U. S. Environmental Protection Agency in 40 CFR Part 792. The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

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Test article dilutions	16 Jul 03	16 Jul 03
Data and Final Report	7 Dec 06	7 Dec 06

To the best of my knowledge, the methods described were the methods followed during the study. The report was determined to be an accurate reflection of the raw data obtained.

Dennis W. Johnson

DENNIS W. JOHNSON
Quality Assurance Coordinator
Toxicology, Aerosol Sciences and
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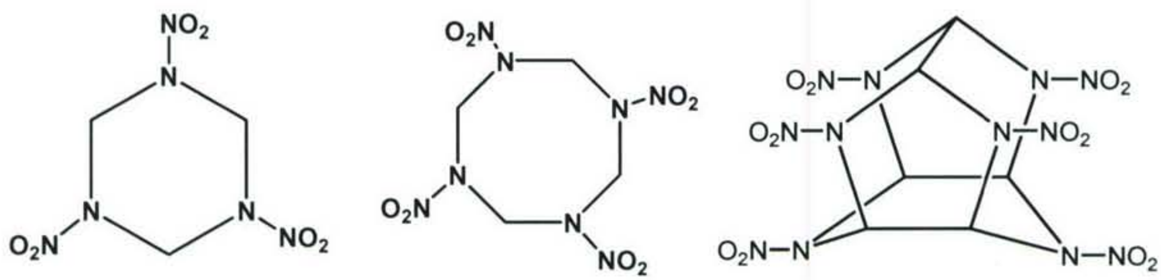
TOXICITY OF THE CYCLIC NITRAMINE ENERGETIC MATERIAL CL-20
TO AQUATIC RECEPTORS

1. INTRODUCTION

Polyazapolycyclic caged polynitramine 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (HNIW or CL-20) is a new energetic material with unknown ecological effects, which requires understanding of potential impacts of its release in the environment. It was originally synthesized by Nielsen *et al.*[1, 2] and is being considered as a potential replacement for existing high explosive and propellant materials [hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)[3]]. Similar to RDX and HMX, CL-20 contains N-NO_2 functional groups (Figure) and was hypothesized to have similar effects on ecological receptors. In contrast to RDX and HMX, CL-20 has a caged structure and has a higher energy output during thermal decomposition.[4] The CL-20 was obtained from ATK Thiokol Propulsion (Ogden, UT, USA, CAS No. 135285-90-4, purity 99.3%).



RDX **HMX** **CL-20 (HNIW)**

Figure. Chemical Structures of the Three Cyclic Nitramines RDX, HMX, and CL-20

There is a need for the development of scientifically based ecotoxicological benchmarks that can be used in Ecological Risk Assessment should aquatic habitats become contaminated with CL-20 through its introduction into the environment. This research was conducted as part of a larger project aimed at advancing the knowledge of the CL-20 fate, transport, and toxicity to ecological receptors.

2. MATERIALS AND METHODS

2.1 Soil Amendment.

A natural soil, Sassafras Sandy Loam (SSL) (Fine-loamy, siliceous, mesic Typic Hapludult) was used in this study to assess CL-20 toxicity to aquatic receptors using water extracts of SSL soils amended with various concentrations of CL-20. This experimental approach will simulate the potential toxicity of CL-20 resulting from its release into the aquatic environment from contaminated surface soil runoff.

This soil was selected for developing ecotoxicological benchmarks because it has physical and chemical characteristics supporting relatively high bioavailability of CL-20 (low organic matter and clay contents). The SSL soil was collected from an open grassland meadow located at the U.S. Army Aberdeen Proving Ground (APG; Edgewood area, MD).

The vegetation and organic horizon were removed to just below the root zone, and the top 12 cm of the A horizon were then collected. The soil was sieved through a 5-mm mesh screen, air-dried for at least 72 hr and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then stored at room temperature. Soil was analyzed for physical and chemical characteristics, and the results of these analyses are presented in Table I.

Table 1. Physical and Chemical Characteristics of Sassafras Sandy Loam Soil

Soil Parameter	Sassafras Sandy Loam
Sand %	69
Silt %	13
Clay %	17
Texture	Sandy loam
CEC cmol kg^{-1}	5.5
Organic matter %	1.2
pH	5.2

The CL-20 was dissolved in acetone and pipetted onto a 2.5 cm thick layer of SSL soil to make an initial soil concentrate. The volume of solution added at any one time did not exceed 15% (v m^{-1}) of the dry soil mass. The acetone was allowed to volatilize in a chemical hood overnight (minimum of 18 hr) in the dark to prevent photolysis of CL-20. Amended soil was transferred into a 1-gal container and mixed for 18 hr on a three-dimensional rotary mixer. The amended soils were stored dry until extracted as described below.

2.2 Weathered and Aged SSL.

Samples of amended SSL soil (100 mg CL-20 kg^{-1} soil) were subjected to weathering and aging for 482 days. Amended soil was placed on a glass tray and wetted with de-ionized water [60% of Water Holding Capacity (WHC), WHC=18% wt of soil] once a week to simulate rain events. At the end of the weathering and aging process, the soil was water extracted as described below. Toxicity Limit tests were conducted on the extracts using ceriodaphnia and algae.

2.3 Modified Toxicity Characteristic Leachate Procedure.

The Toxicity Characteristic Leaching Procedure (TCLP) was adapted for conducting aquatic toxicity tests by replacing the extracting solutions. Typically the TCLP uses acetic acid for extracting materials from soils. We used well water (media the toxicity tests are conducted in) to extract CL-20 from SSL. Aquatic toxicity bioassays, using extracts produced from soil amended with CL-20, were prepared by adding 625 g of amended soil to 2500 mL of test media in 1-gal glass jars. The jars were placed into an end-over-end mixer for 18 hr. After thorough mixing, the contents of the jars were allowed to settle for 1 hr. The supernatant was decanted and centrifuged for 20 min at 5000 rpm (4424 x G) to clear the suspended material enough to be able to see the organisms during testing. The resulting soil extracts were bubbled

with air to raise the pH and dissolved oxygen to appropriate levels. Extracts were then stored in the dark at 4 °C. Extracts were not diluted for use in toxicity testing. The extracts were prepared from soils amended with nominal CL-20 concentrations of 5, 7.5, 10, 20, 30, 40, 50, and 100 mg kg^{-1}.

2.4 Direct Amendment into Water.

The chronic toxicity of CL-20 to ceriodaphnia and fathead minnow in directly-amended aquatic media were investigated by using serial dilution of CL-20 stock solution to produce target exposure concentrations. A flask with stock solution of CL-20 (prepared using fish/ceriodaphnia media) was agitated and placed into a sonic water bath for approximately 8 min. Ultrasonic treatment created a uniform suspension. The stock solution then filtered through a 0.45-µm filter and diluted with media to appropriate concentrations. For the algae toxicity assays, a stock solution of CL-20 was prepared (using algae media) and diluted with media to the appropriate concentrations (the CL-20 stock was not filtered).

2.5 Soil Column Leachate.

Complimentary studies were conducted investigating the transport and fate of CL-20 in soil. The leachate produced form the columns was subjected to 7-Day Ceriodaphnia Reproduction and Survival Toxicity Assays. Controlled Environment Soil-core Microcosm Unit (CESMU) was used to conduct the transport and fate studies over a period of 35 weeks. Intact soil samples were taken from the same sampling area as the previous SSL soil (Aberdeen Proving Ground, MD) and used in the CESMU. An important CESMU feature is the application of tension at the bottom of each soil column (using a vacuum system set at 1/3 bar) to mimic field conditions and prevent artificial build-up of water within columns. This feature requires placement of a controlled-pore porous ceramic plate at the bottom of intact soil core to further approximate physical environment of undisturbed soil during application of tension. The SSL soil cores were amended atop the soil surface by adding 1-cm of soil containing either 100 or 10,000 mg kg^{-1} nominal concentration in freshly amended SSL. Synthetic rainwater [5,6,7] was administered using a peristaltic pump at an application rate of 2.54 cm h^{-1} (1"/h) two times each week for 6 weeks and daily during the last week preceding each harvest to allow sufficient quantity of leachate required for aquatic toxicity testing with ceriodaphnia. Each column received 1.3 L of precipitation every 7 weeks. Ceriodaphnia assays were conducted on leachate produced from weeks 7, 14, 21, 28, and 35.

2.6 CL-20 Analytical Determinations.

The aquatic extract solutions (from ATCLP) were prepared for analytical determination of CL-20 by mixing 5 mL of extract with 5 mL of acidified acetonitrile in glass vials. The solutions were then vortexed and filtered through 0.45-µm Millipore PTFE syringe filters using disposable syringes (10 mL). The first 3 mL of filtrate was discarded before transferring 1 mL of the remaining filtered solution to an HPLC vial. The filtered samples were stored in the refrigerator at 4 °C if not analyzed on the same day.

The CL-20 concentration in the extracts were analytically determined using an HPLC-UV system consisting of an Agilent 1100 HPLC Series equipped with a Supelcosil LC-CN column (25 cm x 4.6 mm x 5 µm), employing an isocratic 70:30 methanol:water mobile phase with a flow rate of 1.0 mL min^{-1} and a 50 µL injection volume. The autosampler was set at 10 °C. Detection of CL-20 was accomplished using a diode array detector at 230 nm wavelength.

Calibration standards were made from intermediate acetonitrile stock solutions with acidified water solution (50:50) to yield standards of 25, 10, 1, 0.25, and 0.05 mg L^{-1} (CL-20/ACN/H_3O^+). Calibration curves were created (r^2>0.9999) with an instrumental limit of detection (LOD) of 0.01 mg L^{-1} (S/N=3). Over five months, the reproducibility of the slope was determined to be 149.0 ± 5.0 with a %RSD of 3.4 (n=14).

2.7 Toxicity Assays.

2.7.1 Algal Growth Assays.

Algal Growth Inhibition Assay was conducted according to the U.S. Environmental Protection Agency (USEPA) standard method[8] using stock solutions of macro- and micro-nutrients prepared with ASTM Type I water. Stock cultures of the unicellular green algae *Selenastrum capricornutum* were grown in 3-L batches and taken during log phase growth for inoculation of test flasks. The initial algal concentration was 1 x 10^4 cell mL^{-1} per test chamber. The total volume for each treatment and control replicate was 100 mL. The test design consisted of four replicates per treatment and control groups, using 250-mL Erlenmeyer flasks as test chambers. The treatment and control groups were subjected to test conditions for 10 days (static non-renewal). Following inoculation with algae, individual test chambers were randomly placed in an incubator at 25 ± 1 °C, and exposed to 350 ft candles of continuous light. The chambers were shaken by hand twice daily. Cell density determinations were accomplished via the manual microscope counting method (hemocytometer) at 96 hr and 10 days. The green algae *S. capricornutum* was exposed to CL-20 added directly into test media. A stock solution suspension of 100 mg L^{-1} CL-20 was prepared and serially diluted to obtain the nominal treatment concentrations (stock was not filtered before dilution) of 6.2, 12.5, 25, 50, and 100 mg L^{-1}. The resulting measured CL-20 concentrations were 4.7, 10.5, 21.5, 46.2, and 94.2 mg/L. A limit 10-d Algal Growth Assay was conducted using aqueous extract from CL-20 freshly amended and weathered/aged SSL soil with a single nominal CL-20 concentration of 1000 mg kg^{-1}. The measured CL-20 concentrations of aqueous extract produced from 1000 mg kg^{-1} freshly amended and weathered/aged SSL were 8 and 4.3 mg L^{-1}, respectively.

2.7.2 Ceriodaphnia Survival and Reproduction Assays.

Ceriodaphnia Survival and Reproduction Assays were conducted according to the USEPA standard method.[8] The test media was prepared from well water drawn from a 400-ft deep well. The water was conditioned by passing through an in line air injection system, a lime stone bed to adjust pH, a Zeta Sol iron removal system, carbon filtration, particulate filtration, and U.V. sterilization. The water for ceriodaphnia cultures was diluted with ASTM Type I water to 90 ppm (mg L^{-1}) total hardness. Ceriodaphnia cultures were fed an algal mixture of *S. capricornutum, Chlamydomonas reinhardti,* and cerophyl extract. The algae were cultured for approximately 7 days[8] before being harvested and fed to the ceriodaphnia at a concentration of approximately 10^6 cells mL^{-1}. Test chambers consisted of 30-mL plastic beakers containing a total of 15 mL of solution. Ten replicates of each treatment and control group were prepared, with each replicate containing one ceriodaphnia. The test media were renewed and fresh food added daily for 7 days. Mortality, reproduction, pH, and dissolved oxygen measurements were recorded every 24 hr. A diurnal light cycle was maintained at 16-hr light and 8 hr-dark. The light intensity was approximately 90 ft candles. The temperature was maintained at 25 °C. Ceriodaphnia cultures were exposed to CL-20 added directly into test media. A stock solution suspension of 32 mg L^{-1} CL-20 was prepared, filtered through a 0.45-µm filter, and serially diluted to obtain the nominal treatment concentrations. The resulting measured concentrations

in assays having CL-20 directly amended into water were 0.2, 0.4, 0.7, 1.5, and 3.0 mg L^{-1}. Aqueous extracts were produced from SSL amended with nominal concentrations of 5, 7.5, 10, 20, 30, and 40 mg Kg^{-1}. The resulting CL-20 concentrations in the aqueous extracts from amended soil were 1, 1.5, 2.3, 4.3, 5.7, and 7.0 mg L^{-1}, respectively. Ceriodaphnia assays were conducted on leachate from CESMU columns that were produced on weeks 7, 14, 21, 28, and 35. The resulting CL-20 concentrations ranged from 0.02 to 1.7 mg L^{-1} (see Table 4).

2.7.3 Fathead Minnow Survival and Growth Assays.

Fathead Minnow Survival and Growth Assays were conducted according to the USEPA standard method[8] using water drawn from a 400-ft deep well. Adult fish *Pimephales promelas* were maintained in 55-gal glass aquaria equipped with under gravel filtration units. Adults were fed twice daily [once with brine shrimp in the mornings and once with Tetramine flake food in the afternoons]. Water temperature was maintained at 25 °C with a diurnal light cycle of 16 hr light for 8 hr dark. Larvae were collected within 24 hr of hatching and used in the bioassays. The test chambers consisted of 1-L glass jars with screw top lids, containing 400 mL of aqueous test solution. Individual test chambers were randomly positioned on the test table, with room temperature and light cycle maintained as described above. The larvae were placed into test chambers using a large bore plastic pipette and were fed freshly hatched brine shrimp twice daily. Test water was renewed daily by siphoning out the test water and debris using an air tube attached to a 1-mL plastic pipette. Approximately 10% of the solution remained in the jars to allow the larva room to swim freely before the addition of new media. Dissolved oxygen, pH, and mortality were recorded daily up to 7 days. At the conclusion of testing, the larva were euthanized, rinsed in distilled water, and then oven-dried at 100 °C for 2 hr. The dry weights of the larvae were measured to the nearest 0.01 mg. The concentrations used in the fish assays having CL-20 directly amended into water were 0.9, 1.8, 2.8, and 3.8 mg L^{-1}. Aqueous extracts were produced from SSL amended with nominal concentrations of 5, 7.5, 10, 20, 30, and 40 mg kg^{-1}. The resulting CL-20 concentrations in the aqueous extracts from amended soil were 0.6, 0.9, 1.3, 2.6, 3.6, 4.5, and 5.7 mg L^{-1}.

2.8 Data Analysis.

Toxicity data were analyzed using nonlinear regression models described in Stephenson *et al.*[9] Either logistic (Gompertz) model [1] or exponential model [2] had the best fit for data in toxicity tests. The best fit of the lines generated by these models were closest to the data points, the variances were the smallest, and the residuals had the best appearance (i.e., most random scattering). These models were:

$$Y = a \times e([\log(1-p)] \times [C/\mathrm{Ecp}]^{b}) \quad [1]$$
$$Y = a \times e(([\log(1-p)] / \mathrm{Ecp}) \times C) + b \quad [2]$$

where

Y	=	number for a measurement endpoint (e.g., number of juveniles)
a	=	control response
e	=	base of the natural logarithm
p	=	percent inhibition/100 (e.g., 0.50 for EC_{50})
C	=	exposure concentration in test soil
Ecp	=	estimate of effect concentration for a specified percent effect
b	=	scale parameter

The ICp parameters used in this study included the CL-20 concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on reproduction or growth endpoints is the preferred parameter for deriving Eco-SSL values. The EC_{50}, a commonly reported value, was included to enable comparisons of the results produced in this study with results reported by other researchers. The asymptotic standard error (a.s.e.) and 95% confidence intervals (C.I.) associated with the point estimates were also determined.

Analysis of Variance (ANOVA) or *t*-Test was used to determine the NOEC and LOEC values for growth, survival, or reproduction data. A significance level of $p < 0.05$ was accepted for determining the NOEC and LOEC values. All analyses were done using measured CL-20 concentrations. Statistical analyses were performed using SYSTAT 7.0.1.[10]

3. RESULTS

3.1 Algal Growth Assay.

The green algae *S. capricornutum* were exposed to CL-20 added directly into test media. A stock solution of 100 mg L^{-1} CL-20 was serially diluted to analytically determined treatment concentrations of 4.7, 10.3, 21.5, 46.2, and 94.2 mg L^{-1}. Algal growth was affected within the CL-20 concentration range tested after 96 hr and 10 days. The IC_{20} and IC_{50} values are 31(18-45, 95% CI) and 116 (88-143, 95% CI) mg L^{-1}, respectively after the 96-hr exposure, and 65 (43-87, 95% CI) and 86 (76-96, 95% CI) mg L-1, respectively after the 10-day exposure. The No Observable Effects Concentration (NOEC) and Lowest Observable Effects Concentration (LOEC) after 96-hr were 10.3 and 21.5 mg CL-20 L^{-1}, respectively. The 10-d NOEC and LOEC were 46.2 and 94.2 mg L^{-1}.

Ten day Algal Growth Assays were also conducted using aqueous extract from freshly amended SSL soil and weathered/aged soil amended with a single nominal CL-20 concentration of 1000 mg kg^{-1}, which produced aqueous extract containing measured CL-20 concentrations of 8 and 4.3 mg L^{-1}, respectively. The *S. capricornutum* growth was stimulated by 48 % when exposed to extracts from freshly amended SSL, which was a statistically significant result (*t*-Test $p = 0.02$). The NOEC for amended SSL and weathered/aged SSL, respectively, were $\geq$8 mg L^{-1} and $\geq$4.3 mg L^{-1}. See Table 2 for a summary of algal toxicity data.

Table 2. Summary of *Selenastrum capricornutum* (Green Algae) Toxicity Data

Exposure	IC_{20} (95%CI) (mg L^{-1})	IC_{50} (95%CI) (mg L^{-1})	NOEC (mg L^{-1})	LOEC (mg L^{-1})
Direct Amendment				
96-h	31 (18-45)	116 (88-143)	10.3	21.5
10-d	65 (43-87)	86 (76-96)	46.2	94.2
Aqueous Extracts				
10-d	--	--	$\geq$ 8 *	--
Weathered/Aged				
10-d	--	--	$\geq$ 4.3 **	--

Direct Amendment: CL-20 added directly to algae media.
Aqueous Extracts: Water extracts from soil freshly amended with CL-20.
Weathered/Aged: Water extracts from soil amended with CL-20 that was subjected to weathering and aging procedure in soil for 482 days.
* Based on Limit test using water extracts of freshly amended soil containing 1000 mg/L.
** Based on Limit test using water extract of soil containing 1000 mg/L CL-20 that was aged 482 days.

3.2 Ceriodaphnia Survival and Reproduction Assays.

Stock solution suspensions of 32 mg L^{-1} CL-20 (nominal) were prepared using ceriodaphnia media and filtered through 0.45-μ filters. The resulting dissolved CL-20 concentration was 3.0 mg L^{-1}. The filtered stock was serially diluted to 0.2, 0.4, 0.7, 1.5, and 3.0 mg L^{-1} and used in the Ceriodaphnia Survival and Reproduction assays. After 7 days of exposure, the NOEC and LOEC values for reproduction were 0.4 and 0.7 mg L^{-1}, respectively. Concentration-response relationship for neonate production after 7-day exposure were determined by nonlinear regression (Logistic Gompertz) model, yielding the IC_{20} and IC_{50} values (mg L^{-1}) of 1.2 (0.9-1.5, 95% CI) and 1.9 (1.6-2.2, 95% CI), respectively. No mortality occurred for up to 7 days in the media directly amended with CL-20.

Ceriodaphnia Survival and Reproduction assays were also conducted using extracts produced from SSL amended with nominal concentrations of 5, 7.5, 10, 20, 30 and 40 mg kg^{-1} CL-20. The resulting measured CL-20 concentrations in the extracts were 1, 1.5, 2.3, 4.3, 5.7 and 7. After 7 days of exposure, the resulting NOEC and LOEC values for reproduction were 1.0 and 1.5 mg L^{-1}, respectively. Based on neonate production, the IC_{20} and IC_{50} values (mg L^{-1}) determined using the Logistic (Gompertz) model were 1.1 (0.9 – 1.3, 95% CI) and 1.8 (1.6 – 2.0, 95% CI), respectively. There were significant effects on ceriodaphnia survival for those exposed to aqueous extracts having CL-20 concentration from 5.7 to 7.0 mg L^{-1}. Based on survival, the 7-d EC_{50} value was 4.2 (3.5-5.0, 95% CI) mg L^{-1}.

Assays were also conducted using extracts produced from 100 mg kg^{-1} CL-20 weathered and aged in SSL soil. The extract was serially diluted to produce measured concentrations of 0.5, 0.9, 1.8, 2.8, and 3.6 mg CL-20 L^{-1}. Based on neonate production, the IC_{20} and IC_{50} values (mg L^{-1}) determined using the Logistic (Gompertz) model were 1.0 (0.8 – 1.3, 95% CI) and 1.6 (1.4 – 1.8, 95% CI), respectively. The NOEC and LOEC values were 0.9 mg L^{-1} CL-20 and 1.8 mg L^{-1} CL-20, respectively. There was 40% mortality in the 3.6 mg L^{-1} CL-20 treatment group after seven days, however a reliable survival EC_{50} could not be determined. See Table 3 for a summary of ceriodaphnia toxicity data.

Aquatic toxicity tests with *Ceriodaphnia dubia* were conducted using leachates collected from CESMU soil columns amended with nominal 100 and 10,000 mg CL-20 kg^{-1} soil. Leachates from replicate soil columns of individual treatments were combined to produce sufficient quantities of leachates required for each day of testing (water change every 24 hr). The leachates did not require centrifugation and were used directly (not serially diluted). Results from week 7 show that after the 7-day exposure of ceriodaphnia to leachates, there was 100 % mortality in leachates from the nominal 10,000 mg kg^{-1} treatment, and 80 % mortality in leachates from the nominal 100 mg kg^{-1} treatment (both results significantly different from control treatment at $p < 0.05$). There was no reproduction in either of these CL-20 treatment groups. Similar results resulted when using leachates produced from week 14. For results from leachates produced in week 21, ceriodaphnia were again exposed to leachates for 7 days. There was 100% mortality in leachate produced from the nominal 10,000 mg kg^{-1} treatment, and 70% mortality from the nominal 100 mg kg^{-1} treatment (both results significantly different from control treatment at $p < 0.05$). The 100 mg kg^{-1} treatment production of a total of 6 offspring was significantly different from control treatment at $p < 0.05$. Leachate produced from weeks 28 and 35 showed similar results; the mortality from the nominal 100 and 10,000 mg kg^{-1} treatments ranged from 70 to 100% with no offspring produced (results significantly different from control treatment at $p < 0.05$). The ceriodaphnia exposed to control soil leachate showed significant effects ($p < 0.05$) when compared to the growth media control that was conducted in

parallel; these effects were seen due to using synthetic rain water to produce leachate. Similar effects were not seen when using ceriodaphnia media to extract CL-20 from amended soils. See Table 4 for a summary of the aquatic toxicity studies of CESMU leachates. The measured concentrations of CL-20 in the leachates showed an overall increase over time.

Table 3. Summary of *Ceriodaphnia dubia* Toxicity Data (7-Day Exposure)

Exposure	IC_{20} (95%CI) (mg L^{-1})	IC_{50} (95%CI) (mg L^{-1})	NOEC (mg L^{-1})	LOEC (mg L^{-1})
Direct Amendment	1.2 (0.9-1.5)	1.9 (1.6-2.2)	0.4	0.7
Aqueous Extracts	1.1 (0.9-1.3)	1.8 (1.6-2.0)	1.0	1.5
Weathered/Aged	1.0 (0.8-1.3)	1.6 (1.4-1.8)	0.9	1.8

Direct Amendment: CL-20 added directly to media.
Aqueous Extracts: Water extracts from soil freshly amended with CL-20.
Weathered/Aged: Water extracts from soil amended with CL-20 that was subjected to weathering and aging procedure in soil for 482 days.

3.3 Fathead Minnow Survival and Growth Assays.

Stock solution suspensions of 50 mg L^{-1} CL-20 (nominal) were prepared and filtered through 0.45-µm filters. The resulting dissolved CL-20 concentration was 3.8 mg L^{-1}. The filtered stock was serially diluted to 0.9, 1.8, 2.8, 3.8 mg L^{-1} and used in the Fathead Minnow Survival and Growth assays. After 7 days of exposure, the NOEC and LOEC values were 1.8 and 2.8 mg L^{-1}, respectively. Concentration-response relationship for growth after the 7-d exposure, as determined using the Logistic (Gompertz) model, for the IC_{20} and IC_{50} values were 2.0 (0.9-3.2, 95% CI) and 2.7 (2.0-3.3, 95% CI) mg L^{-1}, respectively. The 7-d EC_{50} (mg L^{-1}) for survival was 2.0 (1.7-2.3, 95% CI).

Fathead Minnow Survival and Growth assays were also conducted using aqueous extracts produced from SSL amended with nominal concentrations of 5, 7.5, 10, 20, 30, 40, 50 and 100 mg kg^{-1} CL-20. The resulting measured CL-20 concentrations in the extracts were 0.6, 0.9, 1.3, 2.6, 3.6, 4.5, 5.7 and 7 mg L^{-1}. The NOEC and LOEC values were 1.3 and 2.6 mg L^{-1}, respectively. The resulting IC_{20} and IC_{50} values (mg L^{-1}) based on fathead minnow growth, as determined using the Logistic (Gompertz) model, were 1.4 (0.4-2.4, 95% CI) and 2.9 (2.1-3.7, 95% CI), respectively. There were significant survival effects ($p < 0.05$) on fathead minnows exposed to aqueous extracts having CL-20 concentrations from 3.6 to 7 mg L^{-1}. After 7 days of exposure to aqueous extracts from SSL amended with CL-20, the EC_{50} value (mg L^{-1}) for survival was 3.4 (3.1-3.7, 95% CI).

Assays were also conducted using extracts produced from 100 mg kg^{-1} CL-20 weathered and aged in SSL soil. The extract was serially diluted to produce measured concentrations of 0.5, 0.9, 1.8, 2.8, and 3.6 mg L^{-1} CL-20. The resulting IC_{20} and IC_{50} values (mg L^{-1}) based on fathead minnow growth, as determined by Logistic (Gompertz) model, were 2.2 (1.7-2.6, 95% CI) and 3.0 (2.8-3.3, 95% CI), respectively. The NOEC and LOEC were 1.8 and 2.8 mg L^{-1} CL-20, respectively. See Table 5 for a summary of fathead minnow toxicity data.

Table 4. Ceriodaphnia Exposed to CESMU Leachate. Leachate was produced using synthetic rain formulation and was not diluted for toxicity testing.

Sample	Average Offspring Produced	% Mortality	CL-20 (mg L^{-1})
Soil Control (5-28-04, wk 7)	7.3	10	ND*
100 mg L^{-1} CL-20 Leachate	0	80	0.02
10,000 mg L^{-1} CL-20 Leachate	0	100	1.0
Soil Control (7-16-04, wk 14)	2.1	0	ND
100 mg L^{-1} CL-20 Leachate	0	0	0.25
10,000 mg L^{-1} CL-20 Leachate	0	80	1.23
Soil Control (9-3-04, wk 21)	11.1	0	ND
100 mg L^{-1} CL-20 Leachate	0.6	70	0.35
10,000 mg L^{-1} CL-20 Leachate	0	100	1.30
Soil Control (10-22-04, wk 28)	1.2	10	ND
100 mg L^{-1} CL-20 Leachate	0	93	0.28
10,000 mg L^{-1} CL-20 Leachate	0	100	1.70
Soil Control (12-10-04, wk 35)	20.1	0	ND
100 mg L^{-1} CL-20 Leachate	0	100	0.3
10,000 mg L^{-1} CL-20 Leachate	0	70	1.5

* Not Detected

Table 5. Summary of Fathead Minnow Toxicity Data (7-Day Exposure)

Exposure	IC_{20} (95%CI) (mg L^{-1})	IC_{50} (95%CI) (mg L^{-1})	NOEC (mg L^{-1})	LOEC (mg L^{-1})
Direct Amendment	2.0 (0.9-3.1)	2.7 (2.0-3.3)	1.8	2.8
Aqueous Extracts	1.4 (0.4-2.4)	2.9 (2.1-3.7)	1.3	2.6
Weathered/Aged	2.2 (1.7-2.6)	3.0 (2.8-3.3)	1.8	2.8

Direct Amendment: CL-20 added directly to media.
Aqueous Extracts: Water extracts from soil freshly amended with CL-20.
Weathered/Aged: Water extracts from soil amended with CL-20 that was subjected to weathering and aging procedure in soil for 482 days.

4. DISCUSSION

Aquatic bioassays included direct amendments of CL-20 to test media, and aquatic elutriates from SSL soil amended with various concentrations of CL-20, to reasonably estimate the potential toxicity of CL-20 to aquatic receptors that may result from hypothetical direct releases of CL-20 into the aquatic environment, and from hypothetical exposures to runoff from contaminated soil surfaces, respectively. The exposures of test organisms to aqueous extracts of soil containing CL-20 better represent conditions if surface runoff from fields happens

to contaminate bodies of water or contaminated soil erodes into waterways, while results from direct amendment better represent instances if direct contamination of bodies of water occurs.

Results from definitive toxicity assays with aquatic species showed that green alga *S. capricornutum* was the least sensitive organism to CL-20 toxicity among the three species tested. Algal growth was actually stimulated by exposure to CL-20 up to 8 mg L^{-1}, the highest concentration tested in the 10-d assay with elutriates from CL-20 amended into SSL soil, and these results are in agreement with studies conducted by Gong *et al.*[11]. Gong reported no adverse effect of CL-20 on *S. capricornutum* up to 3.6 mg L^{-1} in the 96-hr assay using only soluble CL-20 exposures. Inhibition of algal growth occurred in our studies only after exposing *S. capricornutum* to CL-20 concentrations exceeding normal aqueous solubility, ranging from 21.5 to 94.2 mg L^{-1} for 10 days.

Similar to RDX and HMX, CL-20 contains $N\text{-}NO_2$ functional groups (Figure 1) and was hypothesized to have similar effects on ecological receptors. However, studies by Peters et al. 1991[12], reported the NOEC and LOEC of RDX to ceriodaphnia to be 3.6 and 6.0 mg L^{-1}, respectively. After 7 days of exposure to 16.4 mg/L RDX, there were no effects on ceriodaphnia survival. In comparison, CL-20 toxicity directly-amended into water (Table 3) in our tests appears to be an order of magnitude more toxic to ceriodaphnia. Divergent to the Peters results, studies conducted by Burton and Turley 1994[13] exposing *S. capricornutum* for 96 hr to RDX concentration up to 36.9 mg L^{-1} have shown the NOEC and LOEC for cell growth to be 0.5 and 4.8 mg/L, respectively. These results indicate that CL-20 is less toxic to algae than is RDX.

Although low toxicity effects of CL-20 on algal growth can in part be interpreted as an indication of relatively low risk of environmental impact from an accidental release of this compound, comprehensive assessment should be considered on a broader ecological scale by also investigating the indirect effects of such release. One such possible indirect effect is the increased risk of algal blooms from stimulated growth of algae in response to CL-20 contamination of water bodies and corresponding depletion of dissolved oxygen, which are detrimental to aquatic ecosystems. Examples abound of highly damaging effects resulting from increased nutrient supply or contamination that leads to eutrophycation of aquatic habitats.

In contrast to the effects on autotrophic *S. capricornutum*, CL-20 was highly toxic to heterotrophic aquatic species *C. dubia* and *P. promelas* exposed either in directly amended test media or in aquatic elutriates from CL-20 amended SSL soil. The toxicity benchmarks for reproduction or growth ranged from 1 to 3 mg L^{-1} for both species, and were quite similar for exposures in directly amended media and in aquatic elutriates from CL-20 amended SSL soil (Tables 3 and 5). Comparison of our results to other studies is not possible at the time of preparation of this report because CL-20 is a new energetic material and no aquatic ecotoxicological data were available for these species in published literature. Our results show that IC_{20} and IC_{50} toxicity benchmarks for *C. dubia* reproduction and *P. promelas* growth were within the range of CL-20 solubility in water (3.16 mg L^{-1} at 20°C[14]). This indicates that relevant ecological receptors can be at risk from exposure to CL-20 from different routes of potential CL-20 input, including direct release into aquatic habitats and from contaminated surface soil runoff.

The transport and fate studies also indicate that CL-20 can be persistent in the vadous zone of soil, even for soil that has low clay and organic matter contents. Furthermore, sorption of aqueous CL-20 is relatively small (K_d = 0.02 to 3.83 cm^3 g^{-1}; approx. 2 for SSL), which results in only slight retardation relative to water movement in soil and enables dissolved

CL-20 to move quickly through unsaturated and saturated sediments[15]. After 35 weeks of CL-20 migrating through the soil column, the toxicity effects remained significant; indicating that the aquatic community would also be affected by perculation of water through a soil system, providing an additional route of CL-20 input.

The high toxicity to heterotrophic aquatic species, potentially stimulating effect on autotrophic algae suggest that a substantial release of CL-20 into either terrestrial or aquatic ecosystems may cause significant ecological damage in affected sites. This information should be considered by the manufacturer, potential users, risk assessors, and future site managers, during proposed periods of transition to CL-20 for military products that currently use energetic cyclic nitramines RDX and HMX.

5. CONCLUSIONS

An understanding of potential environmental impacts of the new energetic material CL-20 is crucial for the development of scientifically based ecotoxicological benchmarks that can be used in Ecological Risk Assessment, should aquatic habitats become contaminated with CL-20 through its introduction into the environment.

Toxicity studies were conducted to assess the effects of CL-20 to aquatic species of ecological significance. This research is part of a larger project aimed at advancing the knowledge of the CL-20 fate, transport, and toxicity to ecological receptors, and assessing the potential of CL-20 for its bioaccumulation in soil organisms that may affect higher-level receptors through trophic chain transfer.

Results of aquatic toxicity assays indicate that relevant ecological receptors may be negatively impacted by exposure to CL-20 if the compound was released into the environment. This risk to aquatic species is significant from its potential direct release into aquatic habitats, and from contaminated surface soil runoff or erosion of contaminated soil into water bodies. Several factors indicate that the release of CL-20 to aquatic ecosystems can potentially pose a significant ecological damage in affected sites. These factors include high toxicity of CL-20 to heterotrophic aquatic species and its potentially stimulating effect on autotrophic algae, which can lead to eutrophycation of aquatic habitats. Ecological impacts of CL-20 on aquatic receptors can be further exacerbated by some of the CL-20 fate characteristics including its apparent persistence in soil, and high mobility of aqueous CL-20.

Overall results of this investigation strongly indicate that accidental release of CL-20 into the environment can have detrimental effects on resident ecological receptors in aquatic environments. This information should be considered by the manufacturer, potential users, risk assessors, and future site managers, during proposed periods of transition to CL-20 for military products that currently use energetic cyclic nitramines RDX and HMX, or the nitroaromatic explosive TNT.

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